Carbon balance, partitioning and photosynthetic acclimation in fruit-bearing grapevine (*Vitis vinifera* L. cv. Tempranillo) grown under simulated climate change (elevated CO_2, elevated temperature and moderate drought) scenarios in temperature gradient greenhouses

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Abstract

Although plant performance under elevated CO_2 has been extensively studied in the past little is known about photosynthetic performance changing simultaneously CO_2, water availability and temperature conditions. Moreover, despite of its relevancy in crop responsiveness to elevated CO_2 conditions, plant level C balance is a topic that, comparatively, has received little attention. In order to test responsiveness of grapevine photosynthetic apparatus to predicted climate change conditions, grapevine (*Vitis vinifera* L. cv. Tempranillo) fruit-bearing cuttings were exposed to different CO_2 (elevated, 700 ppm *versus* ambient, ca. 400 ppm), temperature (ambient *versus* elevated, ambient +4 ºC) and irrigation levels (partial *versus* full irrigation). Carbon balance was followed monitoring net photosynthesis (A_N, C gain), respiration (R_D) and photorespiration (R_L) (C losses). Modification of environment ^{13}C isotopic composition ($\delta^{13}C$) under elevated CO_2 (from -10.30 to -24.93 ‰) enabled the further characterization of C partitioning into roots, cuttings, shoots, petioles, leaves, rachides and berries. Irrespective of irrigation level and temperature, exposure to elevated CO_2 induced photosynthetic acclimation of plants. C/N imbalance reflected the inability of plants grown at 700 ppm CO_2 to develop strong C sinks. Partitioning of labeled C to storage organs (main stem and roots) did not avoid accumulation of labeled photoassimilates in leaves, affecting negatively Rubisco carboxylation activity. The study also revealed that, after 20 days of treatment, no oxidative damage to chlorophylls or carotenoids was observed, suggesting a protective role of CO_2 either at current or elevated temperatures against the adverse effect of water stress.

Running title: Grapevine C balance within climate change context

Keywords: Carbon balance; Climate change; Grapevine; Photosynthesis.

Abbreviations: A_N, photosynthesis; Chl, chlorophyll; C_i, sub-stomatal CO_2 concentration; DW, dry weight; E, transpiration; EEA, European Environmental Agency; ETR, electron transport rate; FW, fresh weight; g_S, stomatal conductance; HI, harvest index; J_{max}, maximum electron transport rate contributing to RuBP regeneration; PAR, photosynthetically active radiation; PI, partially irrigated; PPFD, photosynthetic photon flux density; PSI, photosystem I; PSII, photosystem II; qP, photochemical quenching; R_D, dark respiration; R_L, photorespiration; ROS, reactive oxygen species; RWC, relative water content; RuBP, ribulose-1,5-bisphosphate; T_{amb}, ambient temperature; TGG, temperature gradient greenhouse; TOM, total organic matter; TW, turgid weight; T_{+4}, elevated temperature; Vc_{max}, maximum carboxylation velocity of Rubisco; VPDB, vienna pee dee celemnite calcium carbonate; WI, well irrigated; WSC, water-soluble compound; $\delta^{13}C$, C isotopic composition; Φ_{PSII}, actual photosystem II efficiency; $\Phi_{exc.}$, intrinsic photosystem II efficiency.

Introduction

Carbon dioxide (CO_2) concentration has increased since pre-industrial period from 280 to 401.85 μmol mol^{-1} (ppm) currently (NOAA-ESRL, 2014). It is expected that this value could increase to an atmospheric concentration of between 750 and 1300 ppm for the end of the century, if no corrective measures are taken to constrain emissions (IPCC, 2014). Emissions of greenhouse gases caused by human activities have augmented 70% from 1970 to 2004. If greenhouse gases emissions continue at high levels, temperature is predicted to increase between 1.8 and 6.0 ºC (IPCC, 2014). In fact, annual average minimum temperatures in Spain have increased over the last century by around 1.5 ºC. The expected warming is going to be greatest in summer in South-western Europe. More specifically, according to the European Environmental Agency (EEA), an average rise of 4 ºC is predicted by 2080 and extreme summers like the 2003 are likely to become four times as common in Spain (and Southern Europe). In the other hand, precipitation is projected to decrease, in average, by 22 % for the same period. Therefore, global agricultural production will be profoundly impacted (IPCC, 2007).

Since plants with C_3 photosynthetic metabolism are CO_2 limited, it was expected that any CO_2 increase would lead to higher photosynthetic rates (Long, 1991). In a previous study conducted by Drake et al. (1997) exposure to elevated CO_2 increased photosynthetic rates (A_N) up to 58%. However, in long-term experiments, it has been reported that the initial stimulation of photosynthesis following CO_2 application often does not persist and A_N declines below its maximum potential in an acclimation process described as photosynthetic acclimation or photosynthetic down-regulation (Jifon and Wolfe, 2002; Long et al., 2004; Erice et al., 2006b). Photosynthetic down-regulation can be induced by limitations in stomatal and non-stomatal processes. Limitations in stomatal opening have been described to limit A_N as a consequence of stomatal closure

and the corresponding decreased sub-stomatal CO_2 concentration (C_i) (Sánchez-Díaz et al., 2004). Both stomatal (g_S) and mesophyll conductance reductions mediated by drought decrease the Rubisco CO_2 availability, decreasing the CO_2 concentration in the chloroplasts (Flexas et al., 2002; 2009), and are the major cause for the decreased photosynthesis observed in grapevines under water scarcity (Flexas et al., 2010). When drought progresses, the photochemistry and biochemistry of the photosynthesis can be affected, reducing the grapevine photosynthetic capacity (Flexas and Medrano, 2002; Morales et al., 2006). In fact, non-stomatal limitations may also explain decreases in A_N being the result of reduced light capture (PSII activity) and/or decreased carboxylation of RuBP catalyzed by Rubisco. According to Kalina et al. (1997), inhibition is caused by decreased PSII efficiency as a result of the accumulation of inactive PSII reaction centers and the decrease in light harvesting complexes. Other authors suggest that non-stomatal limitation of photosynthesis is attributable to reduced carboxylation efficiency (Long et al., 2004), or to reduced amount/activity of Rubisco. There are two basic mechanisms by which Rubisco down-regulation occurs. The first mechanism hypothesizes that the reduction in Rubisco content occurs as a consequence of the leaf C build-up (Moore et al., 1999; Aranjuelo et al., 2008; 2009; 2011). According to this theory, when plants exposed to elevated CO_2 have limitations for increasing C sink strength, plants decrease their photosynthetic rates to balance C source activity and sink capacity (Aranjuelo et al., 2008; 2009). From this point of view, the reduction in photosynthetic rates would be conditioned by a plant's ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates), or to expand the storage capacity or growth rate of existing sinks. According to the second mechanism, decreases in Rubisco content may reflect a general decrease of leaf protein due to the relocation of N within the plant (Aranjuelo et al., 2011).

Carbon isotope tracers have proved to be an essential tool to study carbon partitioning in plants exposed to elevated CO_2 (Aranjuelo et al., 2008; 2009). After feeding the plants with the stable isotopes (pulse), isotopes are distributed over the different plant organ network that can be followed by the later elemental analyzer-isotope ratio mass spectrometry (EA-IRMS) analyses. Labeling with $^{13}C/^{12}C$ as tracers and characterization of the distribution of labeled compounds into the different plant organs has provided novel and relevant information in studies determining the flow of C through the plants under elevated CO_2 (Kolb and Evans, 2003; Aranjuelo et al., 2009; Molero et al., 2011). In contrast to gas exchange techniques that provide measurements of photosynthetic rates at a single time, when analyzed in leaf dry matter, C isotopic composition ($\delta^{13}C$) integrates photosynthetic activity throughout the period the leaf tissue was synthesized. Moreover, leaf $\delta^{13}C$ values reflect the interplay among all aspects of plant carbon and water relations and are thereby more useful than plant gas exchange measurements as integrators of whole plant function (Aranjuelo et al., 2009).

Many authors have investigated the effects of CO_2, temperature and water stress independently. Interactive effects of elevated CO_2, water stress and temperature have been rarely examined in the past (Lloyd and Farquhar, 2008). Elevated CO_2 decreases stomatal conductance and transpiration rates (Drake et al., 1997; Del Pozo et al., 2005). This CO_2-mediated behavior may influence plant responses to water stress. Different research groups reported decreased photosynthetic rates due to water stress delayed under elevated CO_2 conditions, and an enhanced drought tolerance under elevated CO_2 (Robredo et al., 2007; 2010). Some studies reported increased photosynthetic rates in response to elevated CO_2 under elevated temperature, but not others (Logan et al., 2010, and references therein). However, the analyses of the CO_2 effect and its interaction with other environmental conditions are of great relevance because the responsiveness of

plants to enhanced CO_2 has been shown to differ with temperature, soil water availability, etc. (Aranjuelo et al., 2006; Erice et al., 2006a). Moreover, different stresses often occur simultaneously in the field, such as high temperatures and drought periods, especially in semi-arid or drought-stricken areas. Investigations, performed on field crops as well as on model plants subjected to combined heat and drought stress, have shown that the combination of these two stresses has a stronger detrimental effect on plant growth and productivity compared to each single stress. Furthermore, some reports indicate that it is not possible to extrapolate plant responses to combined stresses starting from the response derived from a single stress (Rampino et al., 2012).

Carbon balance and photosynthetic acclimation remain largely unexplored in grapevine growing under climatic change conditions. Furthermore, the experimental design of the present work tried to emulate natural environment and also to avoid the effects of other external factors (such as radiation, temperature changes, etc.). This was achieved by using temperature gradient greenhouses (TGG), which allowed us to provide a semi-controlled environment where simulate climate change conditions. Therefore, the aim of this work was to investigate the effects of interacting CO_2, temperature and water availability in photosynthetic responsiveness and C partitioning of *Vitis vinifera* (cv. Tempranillo) plants grown in near field conditions. For this purpose, we proceeded to the characterization of physiological parameters, C/N and $\delta^{13}C$ of roots, cuttings, shoots, petioles, leaves, rachides and berries.

Material and Methods

Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* L. cv. Tempranillo were obtained from an experimental vineyard of the Station of Viticulture and Enology of Navarra (Olite,

Navarra, Spain). Cuttings were selected to get fruit-bearing cuttings according to Mullins (1966) and modified by Ollat et al. (1998) and Santa María (2004). Rooting was made in a heat-bed (27 °C) kept in a cool room (5 °C). One month later, the cuttings were transplanted to 7.5-L plastic pots. Cuttings were planted in plastic pots containing a mixture of peat and perlite (2:1: v/v) and transferred to a greenhouse.

Only a single flowering stem was allowed to develop on each plant during growth. Growth conditions in the greenhouse were 26/15 °C and 40/80 % relative humidity (RH) (day/night) and a photoperiod of 15 h with natural daylight supplemented with high-pressure sodium lamps (SON-T Agro Phillips, Eindhoven, Netherlands), providing a minimum photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} at inflorescence level. Plants were irrigated until veraison with the nutrient solution proposed by Ollat et al. (1998).

Experimental design and temperature gradient greenhouses (TGG)

When plants reached veraison stage, they were transferred to the TGG where they were divided according to the different combinations of CO_2 concentration, temperature and water availability to which they were subjected until grapes complete maturity (reaching 21-23 ºBrix). The design of the TGG was based on those described previously (Aranjuelo et al., 2005a; Erice et al., 2006b; Morales et al., 2014). Two greenhouses were maintained at an ambient CO_2 concentration level (approximately 400 ppm) and the other two were maintained at an elevated CO_2 level (approximately 700 ppm) (Fig. 1). Each greenhouse was divided into three modules, thereby providing different temperature values. The central module was regarded as a transition module and no experimental plants were included in it. In each greenhouse, the inlet module was maintained at ambient temperature and the outlet module was maintained at this

ambient temperature +4 ºC (T_{+4}) (Fig. 1). Inside the greenhouses, the pots were placed in holes made in the soil to ensure natural temperature fluctuations at root zone, thus approximating field conditions (Morales et al., 2014). Well-irrigated plants (WI) were watered until maximum soil volumetric water content. Soil water sensors (Watermark soil moisture sensor, Spectrum Technologies Inc., Illinois) were placed into the pots, and were used to control irrigation. Partially irrigated plants (PI) were watered at 40% of pot capacity. Plants were irrigated with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) or distilled water in order to provide all the treated plants the same amount of nutrients. The pots were rotated daily, within the corresponding greenhouse compartment, to avoid edge effects.

C labeling and sampling

Plant C labeling was conducted parallel with exposure to elevated CO_2 conditions. During this period, the plants exposed to elevated CO_2 conditions were grown in an environment where the isotopic composition of the air ^{13}C ($\delta^{13}C$) of the greenhouses was deliberately modified (–24.93 ‰) to distinguish it from the $\delta^{13}C$ of ambient CO_2 (–10.30 ‰). The CO_2 was provided by Air Liquide (Pamplona, Spain). Air isotopic composition inside the corresponding TGG was collected daily using 50 mL syringes (SGE International Pty Ltd, Ringwood, Vic., Australia) and kept in 10 mL vacutainers (BD Vacutainers, Plymouth, UK). To avoid contamination with the air present in the syringe and the needle, both were purged with nitrogen prior to each sampling. The vacutainers were also over-pressurised with the same nitrogen gas so that the pressure inside the vacutainer was above ambient. The labeling period lasted until the end of the experiment.

Water status

Leaf discs were cut with a calibrated cork borer and the fresh (FW), turgid (TW) and dry (DW) weights were determined. Relative water content (RWC) was calculated as: RWC (%) = [(FW-DW)/(TW-DW)] x 100.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and chlorophyll (Chl) fluorescence measurements (n = 5-6 plants, 2 measurements each) were made on young, fully expanded leaves at the same physiological stage (from 8^{th} to 10^{th} node from the top) inside each greenhouse at the respective growth conditions of CO_2 (i.e., plants grown at current CO_2 were measured at 375 ppm CO_2, whereas those grown at elevated CO_2 were measured at 700 ppm). In some experiments, gas exchange parameters were measured at atmospheric CO_2 concentrations of 375 or 700 ppm in all plants, either grown at current or elevated CO_2 concentrations. Also in other cases, CO_2 response curves were made measuring photosynthesis at different atmospheric CO_2 concentrations, ranging from very low to saturating ones. Measurements were made first at 375 ppm CO_2, and then the atmospheric CO_2 concentration was subsequently lowered in a stepwise manner, set again at 375 ppm CO_2 (used as reference) and finally increased stepwise (Larbi et al., 2006). In all these measurements, temperature was 25 ºC. The measurements were performed using a portable photosynthesis system (GFS-3000, Walz, Germany) with a 3-cm^2 cuvette. Gas exchange parameters (net photosynthesis, A_N; maximum carboxylation velocity of Rubisco, Vc_{max}; maximum electron transport rate contributing to RuBP regeneration, J_{max}; transpiration, E; stomatal conductance, g_S; and sub-stomatal CO_2 concentration, C_i) were measured in early morning under a photon flux density of 1200 µmol m^{-2} s^{-1}. Calculations were made according to Von Caemmerer and Farquhar (1981) and Harley et al. (1992). Gas exchange was also measured in dark-adapted leaves after one night in darkness to obtain dark respiration (R_D).

Chlorophyll fluorescence parameters were measured immediately after the photosynthesis measurements with a fluorescence module (PAM-Fluorometer 3055-FL, Walz, Germany) attached to the photosynthesis equipment. The experimental protocol for analysis of Chl fluorescence quenching was performed according to Morales et al. (2000). Parameters monitored were actual (Φ_{PSII}=(F'$_m$-F$_s$)/F'$_m$) and intrinsic ($\Phi_{exc.}$=(F'$_m$-F'$_0$)/F'$_m$) PSII efficiencies, and photochemical quenching (qP=(F'$_m$-F$_s$)/(F'$_m$-F'$_0$). The electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as Φ_{PSII} x PPFD x 0.84 x 0.5, where PPDF is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII (Ogren and Evans, 1993) and 0.84 as the fractional light absorbance (Morales et al., 1991). Multiplying 0.84 x 0.5 gives a value of 0.42, a value very similar to the α term used by other researchers to calculate ETR, which includes the product of leaf absorbance and the partitioning of absorbed quanta between PSI and PSII and determined as the slope of the relationship between Φ_{PSII} and Φ_{CO2} (i.e., the quantum efficiency of gross CO_2 fixation), obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing <1 % O_2 (Valentini et al., 1995). For grapevine cv. Tempranillo, α was reported to be 0.425 (Perez-Martin et al., 2009). Photorespiration (R_L) was estimated as 1/12(ETR – 4 x (A_N + R_D)), according to Valentini et al. (1995).

Photosynthetic pigments determination

One cm^2 leaf disc was cut with a calibrated cork borer, immersed in liquid N_2 and then stored at -80 ºC until use for photosynthetic pigments determinations. Chlorophyll (Chl) *a*, Chl *b* and total carotenoids were measured. Leaf pigments were extracted with

acetone in presence of sodium ascorbate, filtered through a 0.45 μm filter, and analyzed spectrophotometrically according to Morales et al. (2000).

Total organic matter (TOM) and water-soluble compound (WSC) C isotope composition ($\delta^{13}C$)

Berry, rachis, leaf, petiole, shoot, root and cutting samples were collected the last day of labeling (i.e., last day of the experiment), dried at 60 ºC for 48 h, and analyzed for the C isotopic composition ($\delta^{13}C$) of TOM. One mg of ground sample was used for each determination.

To extract the water-soluble compounds (WSC), leaf samples were lyophilized and then ground to a fine powder. About 50 mg of the fine powder were suspended in 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany), mixed, and then centrifuged at 12,000 g for 5 min at 5 ºC. After centrifugation, the supernatant was heated for 3 min at 100 ºC and afterward the solution was put on ice for 3 min. The supernatant containing the WSC fraction was centrifuged at 12,000 g for 5 min at 5 ºC (Nogués et al., 2004). Supernatant fraction was transferred to tin capsules for isotope analysis.

The $^{13}C/^{12}C$ ratios (R) of plant material were determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode.

The $^{13}C/^{12}C$ ratios (R) of air samples were determined by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). Briefly, water vapor and oxygen from gas samples were removed and the carbon dioxide, argon, and nitrogen

gases were separated by gas chromatography (Agilent 6890 Gas Chromatograph) coupled to an isotope ratio mass spectrometer Delta^{plus} via a GC-C Combustion III interphase (ThermoFinnigan). The column used was a 30 cm x 0.32 mm i.d. GS-GASPRO (J. and W. Scientific, USA). The carrier gas was helium at a flow rate of 1.2 mL min^{-1}. The injection port temperature was 220 ^{o}C. The oven temperature was kept at 60 ^{o}C during the entire run. Injection was conducted in the split mode (injected volume 0.3 mL, split flow 20 mL min^{-1}).

The $^{13}C/^{12}C$ ratios (R) of plant material and air samples were expressed as $\delta^{13}C$ values using international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision of 0.1‰: $\delta^{13}C=(R_{sample}/R_{standard})-1$.

C and N content

Carbon and nitrogen content were determined in dry samples previously ground to powder. One mg samples were stored in tin capsules for TOM analyses. Carbon and N contents were determined at the Serveis Cientifico-Técnics of the University of Barcelona (Barcelona, Spain) using an elemental analyzer (EA1108, Series 1; Carbo Erba Instrumentazione, Milan, Italy).

Statistical analysis

Four TGG were used, two set at current CO_2 concentration and the other two at elevated CO_2 concentration. The design of the experiment was split-split-plot, with three factors (CO_2, temperature and water availability). The experiment was repeated once (two biological replicates, comparing grapevine plants growing at current or elevated CO_2

concentrations in the TGG 1 and 2 (Experiment 1) and in the TGG 3 and 4 (Experiment 2)). The number of plants per treatment was 8, but we used only 5-6 of them as experimental replicates (5-6 plants) and then 2 measurements were made per plant (technical replicates). For statistical purposes, we used the 5-6 (gas exchange and Chl fluorescence) or 3 (other determinations) experimental replicates. Data were first tested using a three-way ANOVA (three factors: (i) CO_2 concentration (ii) temperature, and (iii) water availability; and two levels, (i) 700 ppm CO_2 vs. Amb, (ii) T_{amb} vs. T_{+4} and (iii) WI vs. PI, in order to determine the effects of the treatments and their possible interactions. Differences among groups were tested with the Least Significant Differences (*LSD*) post-hoc test. This test was especially useful when effects of treatments were statistically significant or when interaction between factors was detected (not allowing to conclude about the main effects). Results were considered statistically significant at $p<0.05$. Data are presented as means ± standard error (SE). All these statistical analyses were carried out with the SPSS 15.0 statistical package for windows (SPSS inc., Chicago). Each harvest or sampling date was treated as independent data when statistics were carried out, and therefore comparisons were always made among treatments for a given date.

Results

Gas exchange

Figure 2 shows photosynthetic rates measured in plants grown under current or elevated CO_2 concentrations at the prevailing CO_2 concentration of their respective TGG. After 10 and 20 days of treatment, plants grown under water stress conditions had lower ($p<0.001$) photosynthetic rates regardless of CO_2 concentration and temperature (Fig.

2A and 2B). Differences were not significant, however, in plants exposed to elevated temperature after 10 days of treatment (at both CO_2 levels), and those exposed to current CO_2 and temperature after 20 days of treatment (Fig. 2A and 2B). The impaired photosynthetic rates of water stressed plants were mediated by decreases in stomatal conductance, lowering as a consequence transpiration rates (Table 1). Elevated CO_2 increased ($p<0.001$) photosynthetic rates irrespective of water availability and temperature after 10 days of treatment, except in plants grown under ambient temperature and partial irrigation in which increases were not statistically significant (Fig. 2A). Regardless of temperature and irrigation conditions, no CO_2 effects on photosynthetic rates were observed after 20 days of treatment (Fig. 2B). Except in well-irrigated plants grown under elevated temperature and those well irrigated but grown under current temperature and at the sampling date of 10 days, elevated CO_2 closed stomata and reduced transpiration rates at both sampling dates (Table 1). Obviously, all plants grown under elevated CO_2 had higher sub-stomatal CO_2 concentrations (C_i) than their respective controls (Table 1), indicating that the excess CO_2 had entered the leaf. At 20 days, the ANOVA analysis revealed CO_2 x water availability (g_S and C_i, $p<0.01$), CO_2 x temperature (g_S, $p<0.05$) and water availability x temperature (transpiration, $p<0.05$) interactions (Table 1).

No differences in photorespiration (R_L) were found among treatments after 10 days of imposing the different experimental conditions (Fig. 2C). After 20 days of treatment, plants grown under elevated temperature, elevated CO_2 and partial irrigation had the highest photorespiration rates, significantly ($p<0.01$) different from the rest of treatments (Fig. 2D). There was CO_2 x water availability interaction ($p<0.01$), and thus leaves from plants grown under elevated CO_2 and partial irrigation had increased their photorespiration both under ambient or elevated temperature conditions (Fig. 2D).

Leaves from plants grown under ambient temperature, regardless of water status, respired more under elevated than under current CO_2 concentrations either after 10 or 20 days of treatment ($p<0.001$ under well watered conditions, and $p<0.01$ under partial irrigation at both sampling dates) (Fig. 2E and 2F). When grown under elevated temperature, all leaves respired similarly to control (ambient CO_2, ambient temperature and well irrigated) leaves at both sampling dates (Fig. 2E and 2F). These respiration changes reflect an interaction between CO_2 and temperature ($p<0.001$) at both sampling dates (Fig. 2E and 2F).

The increase in atmospheric CO_2 concentration may compensate the decrease in Rubisco activity experienced by plants growing at elevated CO_2. Therefore, under elevated CO_2 there is usually no decrease of photosynthetic activity, but rather a decrease in photosynthetic capacity (Irigoyen et al., 2014). A general reduction of photosynthetic capacity in plants grown under elevated CO_2 suggests photosynthetic acclimation. This is generally evidenced in comparisons of plants grown at ambient and elevated CO_2 and measured at the same CO_2 concentration, either current or elevated. Therefore, all plants were also measured at either 375 or 700 ppm CO_2 irrespective of the CO_2 concentration they were growing (Fig. 3).

Despite some of the effects of water stress already described from Fig. 2 data that were also observable when measurements were made in all plants at the same CO_2 concentration (Fig. 3), a main difference between Fig. 2 and 3 data is that (i) after 10 days of treatment no CO_2 effects on photosynthetic rates were seen either when measured at 375 or 700 ppm (Fig. 3A and 3C), and (ii) after 20 days of treatment plants grown under elevated CO_2 regardless of water availability and temperature had lower (p ranged from <0.01 to <0.001) photosynthetic rates that those grown under current CO_2 both when measurements were made setting CO_2 concentration at 375 or 700 ppm

(decreases were not significant only in the case of plants grown under ambient temperature and well irrigated after 20 days of treatment and measured at 700 ppm CO_2) (Fig. 3B and 3D). The ANOVA analyses revealed only significant interaction ($p<0.05$) between water availability and temperature after 20 days of treatment when measurements were made at 375 ppm (Fig. 3B).

Maximum carboxylation velocity of Rubisco (Vc_{max}) analyses showed that while after 10 days of elevated CO_2 exposure no significant differences derived from CO_2 and water availability treatment were detected, 10 days later, 20 days treated plants grown under 700 ppm and/or low irrigation conditions showed lower ($p<0.001$) Vc_{max} values (Fig. 4A and 4B). Similarly, maximum electron transport rate contributing to RuBP regeneration (J_{max}) showed that CO_2 exposure derived decreases were only detected after 20 days of exposure to elevated CO_2 conditions (Fig. 4C and 4D). There were CO_2 x water availability interactions ($p<0.01$) after 20 days of treatments with respect to both Vc_{max} and J_{max} (Fig. 4B and 4D).

Leaf relative water content (RWC)

Leaf RWC values found at harvest time ranged between 85 and 91% with no significant differences among treatments (Table 2).

Chlorophyll fluorescence

Despite changes observed in gas exchange properties, no remarkable effects of treatments were found ($p<0.001$) on photosynthetic electron transport rates (ETR) at any sampling date (Table 1). This result was confirmed by measuring Φ_{PSII}, $\Phi_{exc.}$ and qP that in most cases did not change in response to the treatments (Table 1). Φ_{PSII} (10 days, $p<0.05$; 20 days, $p<0.01$), $\Phi_{exc.}$ (20 days, $p<0.05$) and qP (20 days, $p<0.01$) showed CO_2 x water availability interactions (Table 1).

After growing plants 10 days under treatments, the $ETR/A_N+R_D+R_L$ ratio only increased in partially irrigated plants grown under ambient temperature and current CO_2 concentration (Table 1). Elevated CO_2 decreased this ratio in all plants, except in those well irrigated grown under ambient temperature (Table 1). After 20 days of treatment, this ratio increased due to water stress in plants grown under elevated temperature and elevated CO_2 (Table 1). Elevated CO_2 increased the $ETR/A_N+R_D+R_L$ ratio only in plants grown under partial irrigation and elevated temperature (Table 1). All these changes were significant at $p<0.05$.

Photosynthetic pigments

No effects of water stress were observed at any sampling date on Chl *a* and Chl *b* concentrations and on the Chl *a*/Chl *b* ratio (Table 2). Elevated CO_2 decreased transiently (after 10 days of treatment) Chl *a* or Chl *b* concentration in some treatments, but effects disappeared with longer exposures to CO_2 (after 20 days of treatment) (Table 2). No effects of CO_2 were found on the Chl *a*/Chl *b* ratio (Table 2). Water stress reduced total carotenoids concentration in plants grown under ambient temperature and current CO_2 concentration after 10 days of treatment but not after 20 (Table 2). Also, elevated CO_2 decreased total carotenoids concentration in well-irrigated plants grown under ambient temperature at both sampling dates (Table 2). Changes in photosynthetic pigment and CO_2 x water availability interactions, when occurred, were significant at $p<0.05$ (Table 2).

C isotope composition ($\delta^{13}C$)

As a consequence of the C labeling, the $\delta^{13}C$ of TOM of plants exposed to elevated CO_2 conditions was ^{13}C-depleted when compared with the respective ambient CO_2 treatment plants (Table 3). More specific analyses revealed that main shoot, roots, leaves and

berries were the organs where more labeled C was detected. In the other hand, in the rachis, and especially in the cutting, the availability of labeled C was the lowest. However, when considering this, it should also be observed that in plants exposed to elevated temperature and partial irrigation such differences between organs were less marked than in fully watered plants grown under ambient temperature. Isotopic analyses also confirmed the fact that, regardless of the organ analyzed, both elevated temperature and partial irrigation diminished the presence of labeled C in TOM. The ANOVA analyses revealed significant interactions between factors (Table 3). In particular, there were interactions between CO_2 and water availability in the $\delta^{13}C$ of TOM in 4 out of 7 organs tested (i.e., root, main shoot, petiole and leaf; p ranged from <0.01 to <0.001). The interaction between CO_2 and temperature was only observed in roots ($p<0.01$).

Although leaf WSC $\delta^{13}C$ values were similar to the ones corresponding to TOM, they were a little bit less ^{13}C depleted. Interestingly, leaf WSC $\delta^{13}C$ values in all treatments were similar to that of berries. As observed with TOM, elevated temperature and partial irrigation diminished the presence of labeled C (Table 3). In the case of leaf WSC $\delta^{13}C$ values, significant interactions were observed between CO_2 and water availability ($p<0.05$) and between CO_2 and temperature ($p<0.001$) (Table 3).

C and N content

Carbon content analyses showed that the main shoot, followed by the leaves, were the organs with the larger C content, whereas the petiole was the one with the lowest one (Table 3). In general terms, irrespective of the organ analyzed, no CO_2, temperature nor water availability significant effects were detected in C content (Table 3).

On the other hand, leaves, followed by rachis and roots, were the organs with more N content, while the cuttings, berries and petioles showed the lowest N content values

(Table 3). While water treatment and growth temperature did not significantly affect N content of different organs (with the exception of main shoot, organ where in addition a significant ($p<0.05$) interaction between these two factors was observed), plants exposed to 700 ppm CO_2 were the ones with the lowest N content (Table 3). Partial irrigation diminished main shoot N content under elevated CO_2 (Table 3). The only organ in which interaction between CO_2 and water availability was detected was the rachis ($p<0.05$; Table 3).

Regardless of the organ analyzed, exposure to elevated CO_2 increased C/N (Table 4). No clear effects in the C/N ratio were observed with water availability or temperature treatments (Table 4). Half of the organs analyzed (root, leaf and rachis) showed significant interactions between CO_2 and water availability ($p<0.05$), whereas main shoot was the organ showing more type of interactions (i.e., CO_2 x temperature ($p<0.05$), and water availability x temperature ($p<0.01$)) (Table 4).

Discussion

Climate change could influence grapevine physiology. The objective of the present research project focuses on evaluating the effect of climate change (elevated CO_2, elevated temperature and water stress) on grapevine physiology and carbon balance. Carbon balance was followed monitoring carbon gains (i.e., net photosynthesis) and losses (i.e., respiration and photorespiration). All these physiological processes and grape quality are sensitive to some extent to one or several stress factors related to climate change. Grapevine photosynthesis, as in other C_3 species, is limited by CO_2 (Mullins et al., 1992; Bindi et al., 1996). Any CO_2 increase therefore may enhance CO_2 fixation rates (Long, 1991). However, plants may experience photosynthetic acclimation

when exposed to elevated CO_2 in long-term experiments, which decreases photosynthesis capacity below its maximum potential (Jifon and Wolfe, 2002; Long et al., 2004; Erice et al., 2006b).

Although 10 days after the beginning of CO_2 treatment, plants exposed to elevated CO_2 conditions showed higher photosynthetic rates, 20 days after beginning, exposure to 700 ppm CO_2 had no significant effect in photosynthesis measured at growth CO_2 concentration (Fig. 2) and decreased photosynthesis measured either at 375 or 700 ppm CO_2 (Fig. 3), in line with previous reports (Aranjuelo et al., 2005b; Del Pozo et al., 2005; Seneweera et al., 2011) and evidencing photosynthetic acclimation. Causes for such photosynthetic acclimation are under debate (Sanz-Sáez et al., 2010). One hypothesis proposes that it comes from stomatal limitations derived from a leaf conductance reduction in plants grown under elevated CO_2 (Sánchez-Díaz et al., 2004). Alternatively, it may come from metabolic limitations, overall ascribed to diminished Rubisco carboxylation activity (Aranjuelo et al., 2005b; Erice et al., 2006a) and/or reduced levels of this enzyme in elevated CO_2-grown plants (Aranjuelo et al., 2005b). Irrespective of water and temperature treatment, the larger sub-stomatal CO_2 concentrations (C_i) of plants exposed to 700 ppm CO_2 discarded limitations in CO_2 availability as a factor involved in photosynthetic down-regulation. After 20 days of treatment, *in vivo* maximum rates of Rubisco carboxylation (Vc_{max}) were markedly decreased in plants grown under elevated CO_2 and partial irrigation (but not in those well watered) regardless of temperature (Fig. 4), supporting the hypothesis of impaired Rubisco carboxylation activity as origin of acclimation. J_{max}, the *in vivo* maximum rate of electron transport driving regeneration of RuBP, also decreased significantly in all plants exposed to elevated CO_2 after 20 days of treatment (Fig. 4). Although in this study Rubisco content was not determined, the 30-50 % decrease in leaf N content

(Table 3) revealed that the inhibition of Vc_{max} and J_{max} was linked to the lower Rubisco protein content. However, it should not be discarded that, as observed by Pérez et al. (2011), depleted Rubisco activity could have also been affected by the enhancement of Rubisco binding inhibitors. More specific CO_2, temperature and irrigation treatment analyses revealed that while photosynthetic responsiveness to elevated CO_2 was not conditioned by growth temperature, partial watering strongly increased the depletion of Vc_{max}. Interestingly, gas exchange analyses also remarked that the lower photosynthetic rates of water-stressed plants were caused by the lower Rubisco activity and C_i of plants. The fact that Rubisco catalyzes CO_2 and O_2 fixation implies that photorespiration (R_L) diminishes the potential photosynthetic activity of plants. According to Andrews and Lorimer (1987), an increase in atmospheric CO_2 increases the leaf internal CO_2 concentration and the CO_2/O_2 ratio at the Rubisco site, which should favor carboxylation rather than oxygenation of ribulose-1,5-bisphosphate (RuBP) with the consequent increase in photosynthetic rates. The fact that in partially watered plants exposed to 700 ppm CO_2, R_L increased suggests that, opposite to what expected, in those plants R_L enhancement (Fig. 2D) contributed to the photosynthetic down-regulation. Moreover, the fact that elevated temperature did not affect Vc_{max}, J_{max} and C_i confirmed that temperature increase did not alter photosynthetic machinery.

The maintenance of equilibrium between light capture and photochemistry requirements is a key point for the avoidance of reactive oxygen species (ROS; Niinemets and Kull, 2001). Our study showed that photochemical changes were accompanied by similar changes in CO_2 fixation in all treatments, with few exceptions. Only plants treated 20 days with elevated CO_2, elevated temperature and drought had marked and physiologically relevant increases of the electron transport rate (ETR) to photosynthesis (A_N) + respiration (R_D) + photorespiration (R_L) ratio (Table 1). Other

researchers have reported similar results of increased ETR/A_N ratios in grapevines grown under water stress and in the field (Flexas et al., 1999; Flexas and Medrano, 2002). These data indicate that the generation (ETR) and the electrons consumption (A_N+R_D+R_L) could be unbalanced in that case. Excess of electrons over those used in photosynthesis could react with O_2 generating ROS and ultimately could lead to damage to cell constituents. Chl and carotenoids (Table 2), main targets of ROS, were not affected in that treatment. If any oxidative damage was present, as suggested by the increased ETR/(A_N+R_D+R_L) ratio of droughted plants grown under elevated temperature and CO_2, this damage was not strong enough to affect photosynthetic pigments. Only Chl and carotenoids decreases were observed in plants treated 10 days but they recovered with time in 20 days-treated plants.

Although studies conducted in ambient CO_2 concentration conditions highlight the relevance of C sink strength as a key factor limiting plant yield, very little attention has been given to this topic in elevated CO_2 concentration studies. This is a matter of major concern because low C sink strength is a key factor conditioning photosynthetic acclimation and therefore plant yield under elevated CO_2 concentration. When plants exposed to elevated CO_2 concentration have limitations in increasing C sink strength, photosynthetic rates are decreased to balance C source activity and sink capacity (Aranjuelo et al., 2009; 2013). A parameter that may indicate the source/sink balance is the C/N ratio, which increases when plant sink capacity is not strong enough to consume or mobilize carbohydrates. Jifon and Wolfe (2002) observed that the effect of N on photosynthetic performance to elevated CO_2 depends on the balance between the availability and demand for N due to its relationship with biomass allocation and source-sink carbon balance. Low N availability may affect plant growth, and thus the capacity to develop new sinks (Erice et al., 2006b). The 183 and 150 % increase in leaf

C/N detected in leaves exposed to elevated CO_2 (under control and partial irrigation conditions respectively) (Table 4) confirmed that they had problems to adjust C sink/source balance. As mentioned above, development of strong C sinks is essential to avoid leaf carbohydrate build-up.

In a recent study where two wheat genotypes with contrasting harvest index (HI) were exposed to elevated CO_2 concentration, Aranjuelo et al. (2013) confirmed that plants with high grain C demand (high HI) were capable of overcoming photosynthetic acclimation with a consequent increase in yield. However, in the case of plants with low grain C demand (low HI), leaf carbohydrate together with depleted N assimilation induced photosynthetic acclimation and the absence of a CO_2 concentration-derived increase in plant biomass. The $^{12}CO_2$ labeling conducted during the experiment enabled the characterization of C assimilation and partitioning toward all the plant organs until grape maturity. Concerning the veraison-maturity period, obtained ^{13}C isotopic composition $\delta^{13}C$ data highlighted that after 30 days of C-labeling, the main shoot, berries, leaves and root were the more C-labeled organs (Table 3). Carbon partitioning among sinks is regulated by the sink themselves and their ability to import photoassimilates (Patric, 1997; Botas, 2004; Zapata et al., 2004). Although a large C-labeling was expected in leaves and berries, our results revealed that an important fraction of photoassimilates remained in leaves and the rest was partitioned toward storage organs like the stem and roots (Botas, 2004; Zapata et al., 2004). Such results highlight the fact that, as mentioned above, those plants had problems to avoid leaf carbohydrate accumulation. According the C/N ratio (Table 4), the C/N increases under elevated CO_2 were mainly due to N reduction (Table 3).

On the other hand, the low labeling observed in the rachis and especially in the cutting, showed that, at this phenological stage, those plant organs did not have any

recently fixed C sink activity. The low-labeled C sink strength of the rachis was remarkable, especially taking into account that it was recently formed. Such results highlighted that the main C source required for the synthesis of the rachis proceeded from remobilization of pre-labeling C. Interestingly, more specific analyses of water and temperature effect on C management highlighted that, at the leaf, stem and in a lesser extent in petioles, water availability and elevated temperature decreased C-labeling, being the plants exposed to drought and elevated temperature the ones with the lowest labeled C. Such differences could have been explained by the deleterious effect of drought and temperature on CO_2 fixation (Fig. 2 and 3). However, when analyzing those data it should be considered that temperature effect on respiration activity could also be involved. Plants use up to 50% of recently formed C in respiration processes, which means that an important fraction of photoassimilates could have been wasted through respiration processes. This point is especially important in heterotrophic organs such as stem with large respiratory activity. It is also remarkable the fact that partitioning of recently assimilated C toward roots was similar in all the treatments. Such results revealed that under stressful growth conditions, compared with the rest of the organs, those plants invested more C on root development.

20-30 days of growth at elevated CO_2 may result short for investigating the acclimation process in grapevine. However, our study showed that even if in an initial step (10 days), exposure to elevated CO_2 increased A_N, after 20 days, and regardless of temperature and irrigation treatment, exposure to 700 ppm CO_2 induced photosynthetic acclimation. The larger C/N ratio detected in leaves exposed to elevated CO_2 affected negatively Vc_{max} and J_{max} with the consequent inhibition of photosynthetic capacity. Such down-regulation was especially marked under drought conditions. ^{12}C labeling highlighted that although storage organs such as main stem and the root represented

important labeled C sink, the large amount of leaf labeled C confirmed that plants exposed to elevated CO_2 were not capable to develop strong C sinks that would enable the avoidance of leaf carbohydrate build-up. Decreases in photosynthetic pigments were observed only in plants grown 10 days under partial irrigation, elevated temperature and CO_2, but they recovered afterwards. In fact, under most experimental conditions, no oxidative damage to Chls and carotenoids was observed, suggesting a protective role of CO_2 either at current or elevated temperatures against the adverse effects of water stress.

In conclusion, irrespective of water availability and temperature, growing under elevated CO_2 concentration induced photosynthetic acclimation in grapevine. Evidence comes from decreases in photosynthetic capacity (measuring photosynthesis at the same CO_2 concentration either 375 ppm or 750 ppm after 20 days of treatment), decreases in photosynthetic parameters such as Vc_{max} and J_{max} in 20 days-treated plants and increases in the leaf C/N ratio at grape ripeness stage (i.e., after 30 days of treatment). Measuring photosynthetic rates or CO_2 response curves at grape ripeness stage is not recommended because photosynthesis and stomatal conductance are low, due to a veraison-ripeness developmental-related decreasing trend (Salazar-Parra et al., 2012). All these changes can be interpreted as symptoms of photosynthetic acclimation in grapevine.

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Figure legends

Figure 1. Temperature (left panels) and air CO_2 concentration (right panels) data recorded in the temperature gradient greenhouses (TGG) along the experimental period (1 month) where CO_2 concentration was set at 400 (ambient) or 700 (elevated) ppm. Each TGG has one module at ambient temperature and another at ambient temperature +4 ºC (T_{+4}).

Figure 2. Photosynthetic rates (A_N) (A and B), photorespiration (R_L) (C and D) and dark respiration (R_D) (E and F) measured at the CO_2 concentration prevailing in the greenhouse at 10 (A, C and E) and 20 (B, D and F) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO_2 concentrations (ambient or 700 ppm CO_2), two temperature regimes (T_{amb} or T_{+4}) and water availability (WI, well irrigated or PI, partially irrigated). Determinations were conducted at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments ($P<0.05$) based on *LSD* test. When significant, interactions between CO_2 concentration (CO_2), water availability (WA) and temperature (T) are also shown.

Figure 3. Photosynthetic rates (A_N) measured at 375 ppm CO_2 at 10 (A) and 20 (B) days of treatment and measured at 700 ppm CO_2 at 10 (C) and 20 (D) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO_2 concentrations (ambient or 700 ppm CO_2), two temperature regimes (T_{amb} or T_{+4}) and water availability (WI, well irrigated or PI, partially irrigated). Sampling was made at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments ($P<0.05$) based on *LSD* test. When significant, interactions between CO_2 concentration (CO_2), water availability (WA) and temperature (T) are also shown.

Figure 4. Rubisco maximum carboxilation efficiency (Vc_{max}) (A and B) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) (C and D) at 10 (A and C) and 20 (B and D) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO_2 concentrations (ambient or 700 ppm CO_2), two temperature regimes (T_{amb} or T_{+4}) and water availability (WI, well irrigated or PI, partially irrigated). Sampling was made at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments ($P<0.05$) based on *LSD* test. When significant, interactions between CO_2 concentration (CO_2), water availability (WA) and temperature (T) are also shown.

Table 1

Table 1. Stomatal conductance (g_S), transpiration (E), sub-stomatal CO_2 concentration (C_i), electron transport rate (ETR), actual and intrinsic photosystem II (PSII) efficiencies (Φ_{PSII} and $\Phi_{exc.}$ respectively), photochemical quenching (qP) and $ETR/A_N+R_D+R_L$ ratios at 10 and 20 days of treatments in leaves of *V. vinifera* cv. Tempranillo grown under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions CO_2xWA, CO_2xT and WAxT are also shown. CO_2, WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns, * and ** indicate no significant differences, and differences at $p<0.05$ and 0.01 respectively.

Treatments									p		
	$A\_T_{amb}\_WI$	$A\_T_{amb}\_PI$	$A\_T_{+4}\_WI$	$A\_T_{+4}\_PI$	$E\_T_{amb}\_WI$	$E\_T_{amb}\_PI$	$E\_T_{+4}\_WI$	$E\_T_{+4}\_PI$	CO_2xWA	CO_2xT	WAxT
g_S (mmol H_2O m^{-2} s^{-1})											
10 days	116±26 a	80±42 bc	103±35 ab	81±24 bc	77±25 bcd	44±18 d	98±36 ab	56±21 cd	ns	ns	ns
20 days	81±26 bc	58±18 cd	113±32 a	50±24 de	93±28 ab	24±12 e	116±32 a	18±8 e	**	*	ns
E (mmol H_2O m^{-2} s^{-1})											
10 days	2.1±0.4 ab	1.5±0.8 cd	2.2±0.7 a	1.9±0.5 abc	1.6±0.4 bcd	1.0±0.4 e	2.3±0.7 a	1.4±0.5 d	ns	ns	ns
20 days	1.5±0.4 bc	1.1±0.4cd	2.6±0.7 a	1.3±0.6 c	1.8±0.5 b	0.5±0.2 e	2.6±0.7 a	0.5±0.2 de	ns	ns	*
C_i (µmol CO_2 mol^{-1} air)											
10 days	219±47 c	187±90 c	199±52 c	203±34 c	398±27 ab	334±145 b	426±65 a	319±112 b	ns	ns	ns
20 days	175±83 d	179±46 d	221±77 d	173±53 d	491±57 ab	311±28 c	537±45 a	436±121 b	**	ns	ns
ETR (µmol e^- m^{-2} s^{-1})											
10 days	80±15 bc	72±7 c	87±23 abc	82±16 bc	88±24 abc	78±21 bc	104±6 a	95±16 ab	ns	ns	ns
20 days	70±10 cd	62±10 d	101±11 a	75±10 bcd	75±11 bcd	65±5 d	84±24 abc	92±23 ab	ns	ns	ns
Φ_{PSII}											
10 days	0.16±0.03 bc	0.14±0.01 c	0.17±0.04 abc	0.16±0.03 bc	0.18±0.05 abc	0.16±0.04 bc	0.21±0.01 a	0.19±0.03 ab	*	ns	ns
20 days	0.13±0.01 c	0.12±0.02 c	0.20±0.02 a	0.15±0.02 bc	0.15±0.02 bc	0.13±0.01 c	0.17±0.05 abc	0.18±0.05 ab	**	ns	ns
$\Phi_{exc.}$											
10 days	0.46±0.10 a	0.50±0.15 a	0.53±0.06 a	0.53±0.10 a	0.51±0.07 a	0.48±0.10 a	0.47±0.06 a	0.52±0.07 a	ns	ns	ns
20 days	0.53±0.12 ab	0.53±0.18 ab	0.50±0.10 abc	0.58±0.05 a	0.52±0.05 ab	0.42±0.12 bc	0.51±0.03 ab	0.36±0.04 c	*	ns	ns
qP											
10 days	0.35±0.06 ab	0.31±0.12 b	0.33±0.09 ab	0.31±0.06 b	0.35±0.12 ab	0.35±0.14 ab	0.44±0.07 a	0.37±0.09 ab	ns	ns	ns
20 days	0.26±0.06 c	0.26±0.07 c	0.41±0.08 ab	0.29±0,.09 c	0.29±0.06 c	0.32±0.08 bc	0.33±0.12 bc	0.51±0.14 a	**	ns	ns
$ETR/A_N+R_D+R_L$											
10 days	8±2 bc	11±2 a	10±2 ab	12±3 a	7±2 c	8±2 bc	7±2 c	8±2 bc	ns	ns	ns
20 days	6±5 bc	8±7 bc	6±5 c	10±8 bc	7±2 bc	13±5 b	10±5 bc	27±7 a	ns	ns	ns

Table 2

Table 2. Relative water content (RWC), Chl *a* and *b*, and Chl *a* /Chl *b* ratio and total carotenoids at 10 and 20 days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses ± S.E. Different letter indicates significant differences between treatments ($P<0.05$) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions CO_2xWA, CO_2xT and WAxT are also shown. CO_2, WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns and * indicate no significant differences and differences at $p<0.05$ respectively.

Treatments									p		
	A_T_{amb}_WI	A_T_{amb}_PI	A_T_{+4}_WI	A_T_{+4}_PI	E_T_{amb}_WI	E_T_{amb}_PI	E_T_{+4}_WI	E_T_{+4}_PI	CO_2xWA	CO_2xT	WAxT
RWC (%)	89±7 a	87±6 a	89±4 a	89±4 a	91±3 a	88±4 a	90±6 a	85±10 a	ns	*	ns
Chl *a* (µmol m^{-2})											
10 days	525±77 a	445±64 ab	397±9 b	433±77 ab	365±25 b	443±19 ab	380±31 b	334±123b	ns	ns	ns
20 days	426±69 a	343±59 a	405±71 a	329±75 a	298±55 a	400±117 a	337±90 a	387±72 a	*	ns	ns
Chl *b* (µmol m^{-2})											
10 days	175±37 a	169±71 ab	118±8 abc	140±32 abc	101±9 c	126±9 c	114±12 bc	93±37 c	ns	ns	ns
20 days	135±30 a	96±18 ab	114±21 ab	84±46 ab	80±15 b	117±42 ab	89±33 ab	112±22 ab	*	ns	ns
Chl *a* /Chl *b*											
10 days	3.0±0.3 a	2.9±0.8 a	3.4±0.2 a	3.1±0.3 a	3.6±0.3 a	3.5±0.1 a	3.4±0.4 a	3.6±0.2 a	ns	ns	ns
20 days	3.2±0.3 b	3.6±0.1 ab	3.6±0.1 ab	4.7±2.2 a	3.7±0.1 ab	3.5±0.2 ab	3.9±0.5 ab	3.5±0.1 ab	ns	ns	ns
Total carotenoids (µg m^{-2})											
10 days	273±49 a	196±29 b	201±11 b	215±40 b	180±9 b	217±10 ab	191±13 b	165±59b	ns	ns	ns
20 days	207±42 a	171±24 ab	201±31 ab	163±30 ab	146±27 b	193±47 ab	157±39 ab	191±25 ab	*	ns	ns

Table 3

Table 3. Carbon isotopic composition ($\delta^{13}C$, ‰) and C and N content (% of DW) at the end of the treatments (grape ripeness stage) in the different organs of *V. vinifera* cv. Tempranillo grown from veraison to maturity under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on *LSD* test. WSC indicates water-soluble compounds. Significance (p) of the ANOVA analyses for the interactions CO_2xWA, CO_2xT and WAxT are also shown. CO_2, WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns, *, ** and *** indicate no significant differences, and differences at $p<0.05$, 0.01 and 0.001 respectively.

Treatments									p		
	A_T_{amb}_WI	A_T_{amb}_PI	A_T_{+4}_WI	A_T_{+4}_PI	E_T_{amb}_WI	E_T_{amb}_PI	E_T_{+4}_WI	E_T_{+4}_PI	CO_2xWA	CO_2xT	WAxT
$\delta^{13}C$ (‰)											
Root	-25.5±0.6 a	-25.8±1.0 a	-26.2±1.1 a	-26.9±0.4 a	-37.0±0.7 d	-33.1±0.9 c	-34.5±0.4 c	-30.8±0.5 b	***	**	ns
Cutting	-27.0±0.8 ab	-27.0±0.4 ab	-26.5±0.2 a	-26.7±0.4 a	-29.4±0.7 b	-28.2±0.5 ab	-28.3±0.4 ab	-27.7±0.3 ab	ns	ns	ns
Main Shoot	-27.3±0.1 a	-27.4±0.5 a	-26.6±0.3 a	-28.3±0.7 a	-37.6±2.4 d	-32.2±0.4 bc	-34.8±0.7 cd	-31.6±0.2 b	***	ns	ns
Petiole	-27.0±0.4 a	-27.0±0.5 a	-27.7±0.2 a	-27.0±0.6 a	-34.5±0.5 c	-32.4±0.4 bc	-33.9±0.7 c	-30.9±0.1 b	**	ns	ns
Leaf	-28.1±0.1 a	-27.6±0.6 a	-27.7±0.4 a	-27.9±0.1 a	-36.9±0.5 d	-34.1±0.2 bc	-35.5±1.2 cd	-32.9±0.2 b	**	ns	ns
Rachis	-29.1±0.2 a	-28.8±0.7 a	-28.6±0.7 a	-28.8±0.2 a	-32.3±0.1 b	-32.1±0.5 b	-31.4±0.2 b	-30.7±0.2 ab	ns	ns	ns
Berry	-25.8±0.3 a	-25.4±0.3 a	-25.4±0.5 a	-25.3±0.6 a	-36.2±0.8 c	-33.7±0.9 b	-34.4±1.4 bc	-32.6±0.5 b	ns	ns	ns
Leaf (WSC)	-24.2±0.3 a	-23.4±0.5 a	-23.9±0.2 a	-24.0±0.3 a	-36.0±0.5 c	-33.8±0.7 b	-33.6±0.6 b	-31.6±0.2 b	*	***	ns
C (%)											
Root	42.5±0.2 ab	42.2±0.9 ab	44.1±0.3 ab	44.4±0.9 a	41.2±1.5 b	44.0±1.9 ab	44.2±0.5 ab	43.5±0.5 ab	ns	ns	ns
Cutting	44.8±0.5 a	46.3±1.5 a	45.9±0.3 a	45.6±1.7 a	45.1±0.4 a	47.4±1.2 a	46.1±0.2 a	42.1±3.8 a	ns	ns	ns
Main Shoot	48.9±0.7 a	47.2±0.6 abc	45.8±0.3 bcd	45.2±0.8 cd	44.7±1.4 d	47.7±0.4 ab	46.3±1.0 bcd	45.3±0.4 cd	ns	ns	ns
Petiole	38.1±1.9 a	38.7±1.0 a	36.4±2.1 a	37.8±0.8 a	40.7±0.8 a	40.2±1.1 a	39.3±0.7 a	38.0±1.5 a	ns	ns	ns
Leaf	46.9±1.2 a	46.3±1.2 a	46.4±0.4 a	46.8±0.4 a	41.6±3.0 a	47.7±0.8 a	47.3±1.4 a	46.4±0.4 a	ns	ns	ns
Rachis	42.9±1.2 a	42.0±0.2 a	41.6±0.6 a	42.0±0.2 a	41.5±0.4 a	41.5±0.5 a	43.0±1.2 a	42.3±0.3 a	ns	ns	ns
Berry	42.7±3.4 a	40.9±1.7 a	44.1±1.2 a	39.6±0.5 a	43.0±2.2 a	43.5±1.7 a	41.0±1.6 a	42.2±2.7 a	ns	ns	ns
N (%)											
Root	1.17±0.17 bc	1.34±0.17 ab	1.48±0.06 ab	1.64±0.02 a	0.64±0.04 d	0.93±0.13 cd	0.79±0.12 d	1.14±0.09 bc	ns	ns	ns
Cutting	0.62±0.07 a	0.56±0.04 a	0.67±0.06 a	0.66±0.09 a	0.59±0.04 a	0.57±0.11 a	0.50±0.02 a	0.60±0.10 a	ns	ns	ns
Main Shoot	0.87±0.01 bc	0.89±0.05 bc	1.06±0.07 abc	1.22±0.19 a	1.18±0.20 ab	0.78±0.02 c	0.78±0.07 c	1.06±0.05 abc	ns	ns	*
Petiole	0.76±0.04 a	1.00±0.07 a	1.16±0.21 a	1.19±0.15 a	0.68±0.08 a	0.79±0.05 a	0.65±0.09 a	0.97±0.11 a	ns	ns	ns
Leaf	3.12±0.24 ab	3.19±0.15 a	3.10±0.24 ab	3.30±0.08 a	1.50±0.05 d	2.26±0.19 cd	1.94±0.20 cd	2.35±0.03 bc	ns	ns	ns
Rachis	1.92±0.28 b	2.37±0.05 ab	2.39±0.06 ab	3.11±0.23 a	1.99±0.19 b	1.58±0.06 b	2.13±0.42 ab	2.21±0.15 ab	*	ns	ns
Berry	0.68±0.07 b	0.70±0.01 b	0.71±0.05 b	0.87±0.05 a	0.61±0.04 b	0.66±0.03 b	0.59±0.06 b	0.67±0.05 b	ns	ns	ns

Table 4

Table 4. C/N ratio at the end of the treatments (grape ripeness stage) in the different organs of *V. vinifera* cv. Tempranillo grown from veraison to maturity under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses ± S.E. Different letter indicates significant differences between treatments ($P<0.05$) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions CO_2xWA, CO_2xT and WAxT are also shown. CO_2, WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns, * and ** indicate no significant differences, and differences at $p<0.05$ and 0.01 respectively.

Treatments	$A\_T_{amb}\_WI$	$A\_T_{amb}\_PI$	$A\_T_{+4}\_WI$	$A\_T_{+4}\_PI$	$E\_T_{amb}\_WI$	$E\_T_{amb}\_PI$	$E\_T_{+4}\_WI$	$E\_T_{+4}\_PI$	p CO_2xWA	p CO_2xT	p WAxT
C/N											
Root	38.0±6.0 cd	32.3±3.1 d	29.9±1.2 d	27.1±0.8 d	64.2±1.7 a	48.6±4.5 bc	58.4±7.4 ab	38.6±2.5 cd	*	ns	ns
Cutting	73.9±9.6 a	83.0±5.6 a	69.4±5.7 a	71.4±9.0 a	77.1±5.6 a	89.5±16.3 a	92.0±3.1 a	72.7±7.0 a	ns	ns	ns
Main Shoot	56.3±1.4 ab	53.6±3.6 abc	43.6±2.8 bcd	39.2±6.5 d	40.2±6.9 d	61.3±1.8 a	60.6±5.9 a	42.8±1.9 cd	ns	*	**
Petiole	50.6±4.8 ab	39.0±3.5 bc	33.4±4.2 c	33.1±5.4 c	61.9±7.7 a	51.2±2.8 ab	63.2±8.8 a	40.2±4.8 bc	ns	ns	ns
Leaf	15.1±0.8 d	14.6±0.3 d	15.2±1.2 d	14.2±0.5 d	27.7±1.3 a	21.5±2.3 bc	24.8±1.7 ab	19.8±0.2 c	*	ns	ns
Rachis	23.4±3.5 ab	17.8±0.4 bc	17.4±0.5 bc	13.7±1.0 c	21.2±2.0 ab	26.4±0.7 a	21.5±3.6 ab	19.3±1.2 bc	*	ns	ns
Berry	63.6±6.8 ab	58.7±1.9 b	62.8±4.5 ab	45.8±3.0 c	70.9±3.0 a	65.9±1.7 ab	70.9±4.7 a	63.4±0.8 ab	ns	ns	ns

Figure 1

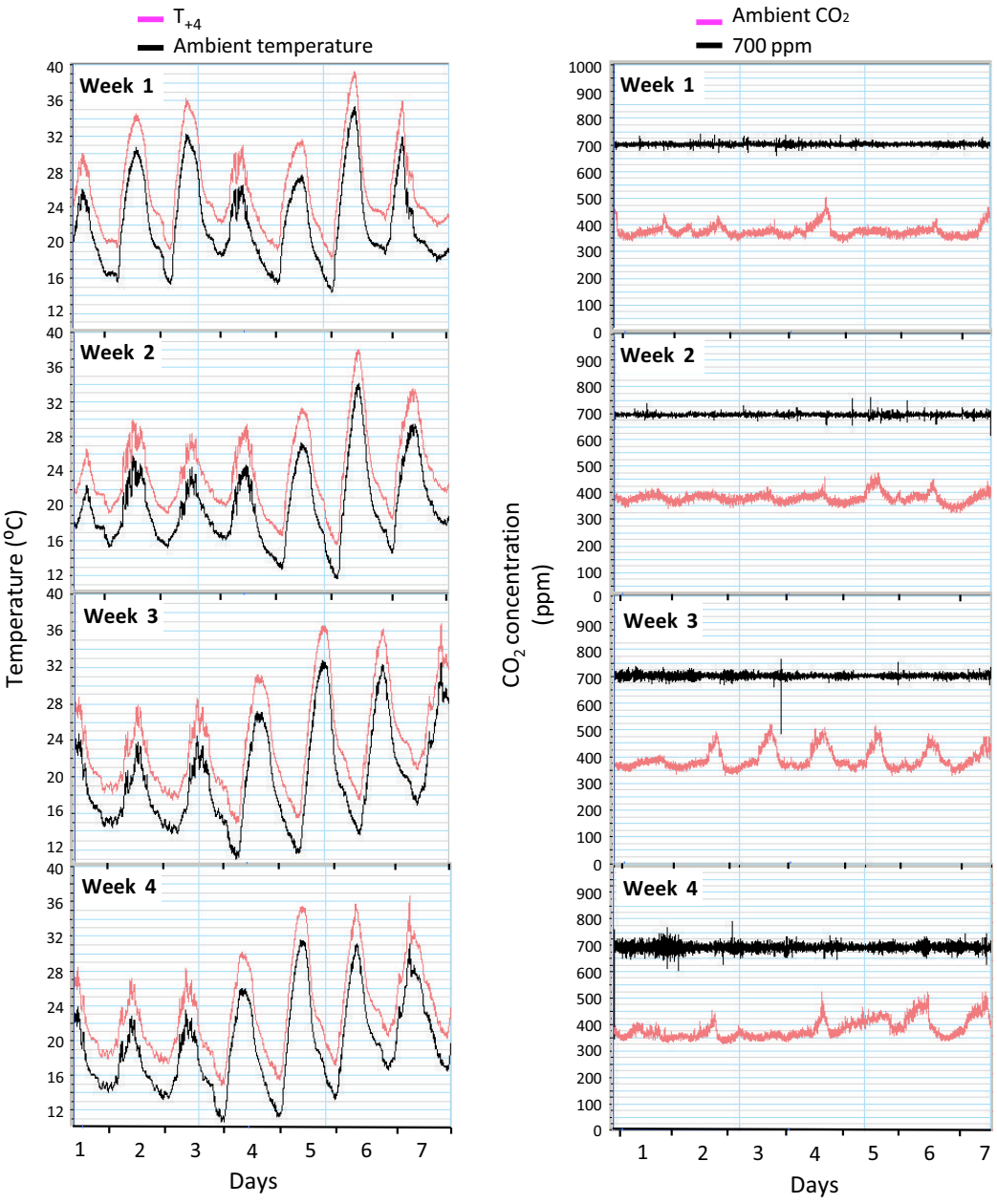
T+4
Ambient temperature
Week 1
Week 2
Week 3
Week 4
Temperature (°C)
Days
Ambient CO2
700 ppm
CO2 concentration (ppm)

Figure 2

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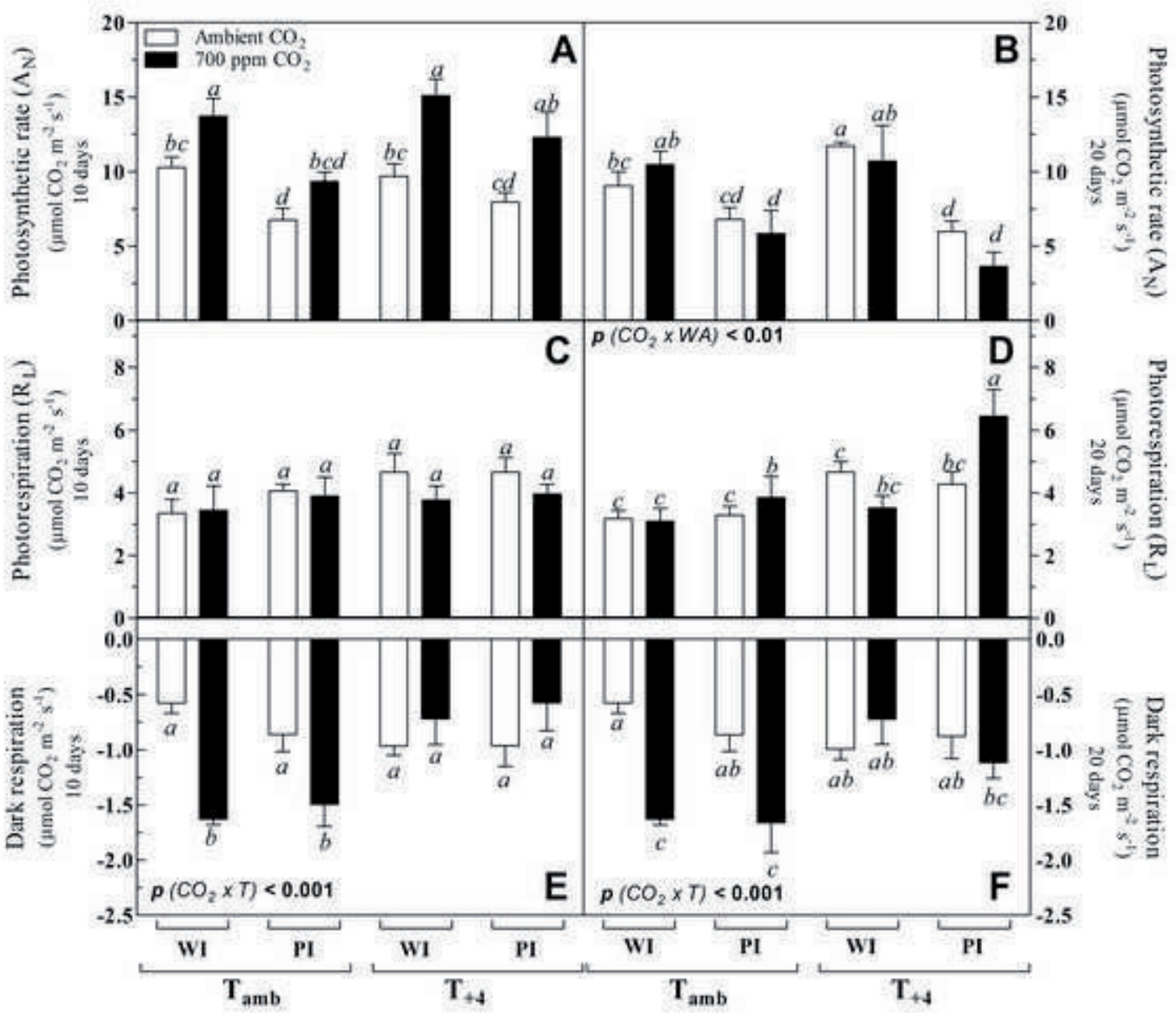

Figure 3

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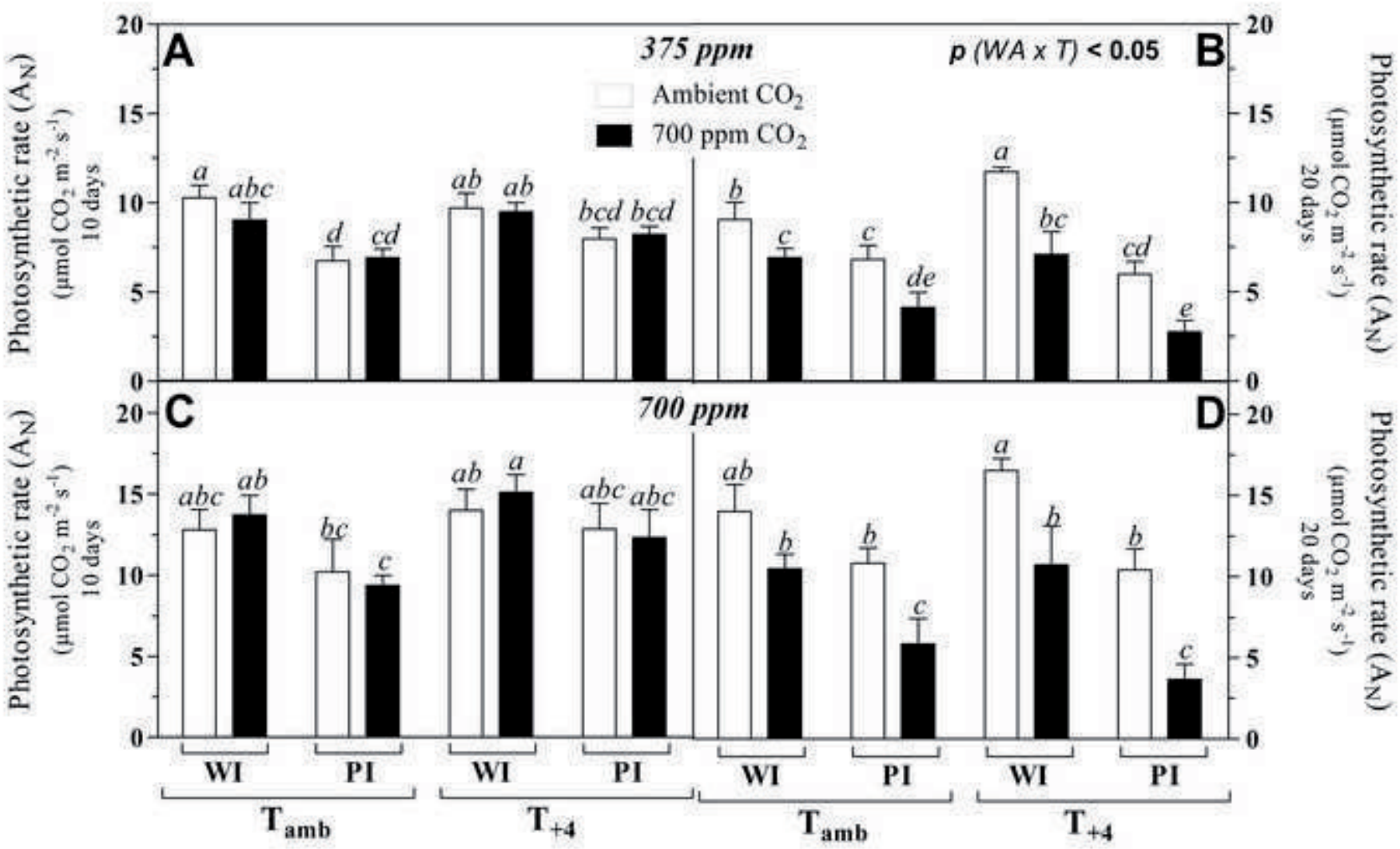

Figure 4

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